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Comparative phylogeography of two troglobitic Coleoptera (Leiodidae, Leptodirini) species from Romania based on mitochondrial DNA

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Abstract

About 50 species of cave-obligate Leptodirini (Leiodidae) beetles have been described so far in Romania, most of them populating caves in the Apuseni Mountains (north-western Romania) and the Southern Carpathians. In this contribution, we present the first molecular phylogeographic study of the two troglobiotic *Pholeuon* species from the Apuseni Mountains. The two species are *Pholeuon* (s.str.) *leptodirum* and *Pholeuon* (*Parapholeuon*) *gracile*, endemic to Bihorului Mountains and Pădurea Craiului Mountains, respectively. To examine the genetic divergence within and between the two species we sequenced 571 bp of the mitochondrial COI gene in a total of 145 specimens, 56 specimens of the first species (collected in five caves) and 89 of the second species (collected in eight caves) across their geographic ranges. We found very low genetic variation, four haplotypes in *P. leptodirum* and seven haplotypes in *P. gracile*, and a maximum of 0.7% and 0.9% intraspecific divergence, respectively. However, a significant genetic divergence of 6.55% was found between species. The results are consistent with previous definitions of the two species based on morphological characters, while caution should be taken

* These authors had an equal contribution.

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in considering attributions to different subspecies. Our research contributes to the phylogeographic information of troglobitic beetles, providing a solid basis for future comparison with other terrestrial or aquatic cave adapted species.

Keywords
Carpathians, cave beetles, cytochrome oxidase gene I, *Pholeuon*, population genetics

Introduction

A detailed knowledge of the biology and ecology of the species and their genetic structure at the population level plays a crucial role in minimizing the effect of loss of biodiversity. Important target of global conservation efforts are endemic species. These, with their specific climatic and environmental requirements and generally limited dispersal capacity, are particularly vulnerable to extinction (Myers et al. 2000; Lamoreux et al 2006). Obligate subterranean species, cave-adapted so called troglobionts, are particularly vulnerable to the pollution produced on the surface, which percolates soil and layers of limestone, contaminating the subterranean habitats (Wood and Perkins 2002; Manenti et al. 2021). Subterranean environments are inhabited by a specialized fauna living in relatively stable conditions that creates a unique biological laboratory where evolutionary and ecological processes can be studied in situ (Mammola 2019). Subterranean terrestrial habitats are characterized by absence of light, saturated air humidity, constant temperatures and often scarce food resources (Howarth et al. 2008). The ecological balance is, therefore, fragile and any disturbance can potentially cause alteration of the natural conditions, fragmentation of the habitat and populations, with the possible extinction of the troglobitic species.

Romania hosts many unique karst landscapes and caves, and several types of endemism are found in its subterranean fauna. The occurrence and distribution of the Romanian cave fauna can be to a larger extent explained by paleogeography and ecology of the group (Moldovan 2008). Among them, the Leptodirini (Coleoptera, Leiodidae) species include strictly cave-adapted beetles and represent a conspicuous group of species endemic to one or a few caves in a karst area. Indeed, eight Leptodirini genera with 49 endemic species inhabit Romanian caves (Moldovan et al. 2020). Their limited distribution in a fragmented karst landscape make Romanian Leptodirini a model group for studying speciation and historical biogeographic processes both at fine and large scales. Molecular studies on Leptodirini species are still very limited in number, and the most comprehensive study was undertaken by Ribera et al. (2010); this comprised 57 Leptodirini species from all the major lineages distributed in the Western Mediterranean area, including two Romanian species. The molecular clock approximation suggested that the main Western Mediterranean lineages originated in Early-Mid Oligocene and that the ancestral species were already present in the geographical areas in which they are found today (Ribera et al. 2010).

Romanian Leptodirini group is considered to derive from the Dinaric ancestors, before the separation of the Carpathian Mountains from the Dinarides (Jeannel 1931; Decu

and Negrea 1969; Moldovan and Rajka 2007). The Romanian Carpathians are divided into three geographical units: The Apuseni Mountains (North-West), the Eastern Carpathians, and the Southern Carpathians. The Apuseni Mountains are characterized by an amazing variety of relief micro forms, both above and underground, and a considerable number of caves (3,960 caves, with a cave density of 3.5 caves/km^2) (Cocean 2000). The unique features of the fragmented karst in this region, interrelated with the underground hydrological network, could present a great opportunity for the dispersal of the species. Whereas the complex geological settings, from local to regional context, potentially function as geographical barriers for the dispersal of subterranean fauna (Moldovan 2008).

Romanian Leptodirini were studied, so far, for various aspects: ecological, taxonomic, either by classical taxonomy (the revision of genus *Drimeotus*; Moldovan 2000) and by morphometrics (Racoviță 1996; Racoviță 1998–1999; Racoviță 2010, 2011), historical biogeography (Moldovan and Rajka 2007), cytogenetic (Buzilă and Marec 2000), molecular phylogeography (Bucur et al. 2003). Studies on molecular phylogeography of Leptodirini were carried out on the phyletic series of *Drimeotus* with three genera, *Drimeotus*, *Pholeuon*, and *Protopholeuon*, all three endemics to Apuseni Mountains (Bucur et al. 2003). *Drimeotus* and *Pholeuon* include three and two subgenera, respectively, while *Protopholeuon* is a monospecific genus. Species belonging to *Pholeuon* and *Protopholeuon* are considered more troglomorphic (sensu Christiansen 2012) (longer appendages, slender body) than *Drimeotus* (Moldovan et al. 2007). The study of Ribera et al. (2010), including representatives from Romanian Carpathians, established the monophyly of the phyletic series *Drimeotus*. On the contrary, the subgenera belonging to *Drimeotus* and *Pholeuon* genera were not monophyletic (Bucur et al. 2003), offering multiple independent colonization events by surface ancestors as a possible explanation for their actual distribution.

In the present study we carried out a comparative phylogeographic analysis of the two cave adapted Leptodirini subgenera from the Apuseni Mountains. In particular, we considered two mountain ranges, Pădurea Craiului and Bihorului, with the species belonging to their corresponding endemic subgenera: *Pholeuon* (*Parapholeuon*) and *Pholeuon* (s. str). Until present, three species have been described from each subgenus, *Parapholeuon* with *P. gracile*, *P. moczaryi*, and *P. angustiventre* and *Pholeoun* s. str. with *P. angusticolle*, *P. knirschi* and *P. leptodirum*. For this study, one species from each subgenus, *P. gracile* and *P. leptodirum*, both including several populations/subspecies, were considered. The analyses included all three described subspecies of *P. gracile* while only five of eleven described subspecies of *P. leptodirum* were included (Racoviță 2011). The description of subspecies is rather controversial as it is solely based on morphometric characters, and their taxonomical status reaches beyond the scope of this paper.

The main aims of the present study were: i) to test the congruence between the subspecies identified on the basis of morphometric measurements and the putative molecular species delimited using DNA barcoding (mitochondrial cytochrome oxidase subunit I, COI); and ii) to investigate the degree of genetic differentiation both within and between the two cave-adapted species, and to analyse it according to the geographic distribution of populations.

Materials and methods

All analyzed populations are endemic in two massifs of the Apuseni Mountains, Bihorului and Pădurea Craiului, and have limited distribution in one or few caves only (Fig. 1 and Table 1).

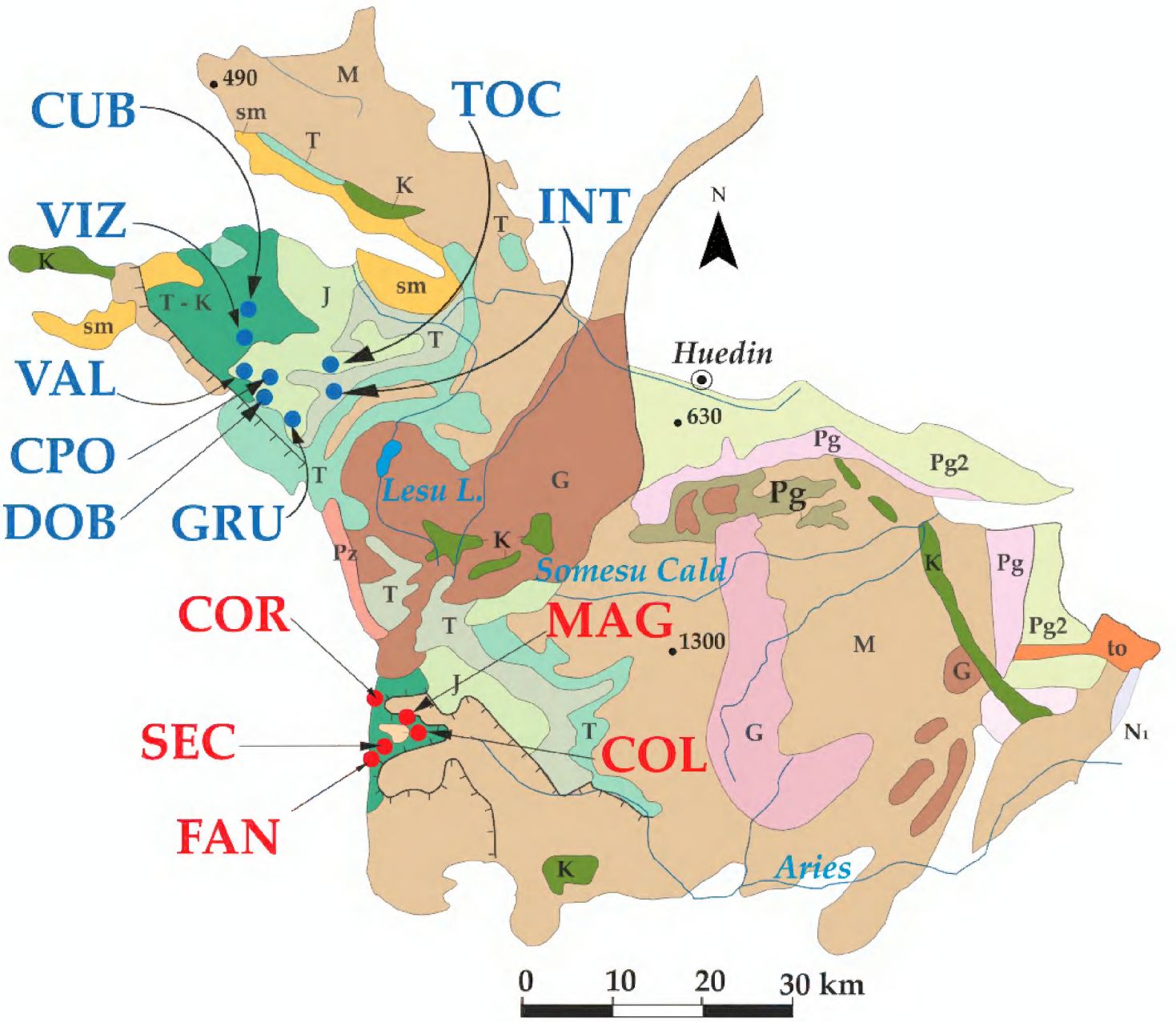

Figure 1. Sampling sites of *P.* (*Parapholeuon*) *gracile* in Pădurea Craiului Mountains (blue) and *P.* (s. str.) *leptodirum* in Bihorului Mountains (red). Codes refer to the caves' name, as indicated in Table 1.

Table 1. Leptodirini subspecies (as proposed by Racoviță 2011) included in the study with the codes used throughout the paper, the hydro-karstic basins, altitude and slope where the caves are located. Sample size (N), haplotypes and number of specimens sharing each haplotype are also indicated.

Cave Name	Code	River valley	Basin	Subspecies	Altitude (m a.s.l.)	Geographic slope	N	Haplotype (no. specimens)
Pădurea Craiului Mountains – *P.* (*Parapholeuon*) *gracile*								
Cubleș	CUB	Vida	Holod	*P. g. gracile*	440	right	11	H1 (9), H2 (2)
Vizu II	VIZ	Vida	Holod	*P. g. bokorianum*	350	left	12	H3 (5), H6 (6), H5 (1)
Tocoș	TOC	Runcșor	Roșia	*P. g. chappuisi*	585	right	11	H1 (8), H2 (2), H7 (1)
Întorsuri	INT	Runcșor	Roșia	*P. g. chappuisi*	575	right	11	H1 (9), H2 (2)
Ciur Ponor	CPO	Albioara	Roșia	*P. g. chappuisi*	510	left	10	H3 (10)
Doboș	DOB	Albioara	Roșia	*P. g. chappuisi*	465	left	11	H3 (11)
Vălău	VAL	Albioara	Roșia	*P. g. chappuisi*	355	right	12	H3 (9), H4 (3)
Gruieț	GRU	Șteazelor	Roșia	*P. g. chappuisi*	300	left	11	H1 (9), H2 (2)
Bihorului Mountains – P. (s.str.) leptodirum								
Coliboaia	COL	Sighiștel	Sighiștel	*P. l. jeanneli*	560	right	11	H8 (11)
Măgura	MAG	Sighiștel	Sighiștel	*P. l. hazayi*	550	right	12	H8 (12)
Corbasca	COR	Sighiștel	Sighiștel	*P. l. moldovani*	500	left	11	H8 (6), H9 (5)
Fânațe	FAN	Bulzului	Crișul Băița	*P. l. leptodirum*	560	right	10	H8 (8), H11 (2)
Secătura	SEC	Bulzului	Crișul Băița	*P. l. problematicus*	1080	right	12	H10 (12)

Sampling

Specimens of *P. gracile*, belonging to the three described subspecies (*P. gracile* s. str., *P. g. chappuisi*, and *P. g. bokorianum*), were collected from eight caves located in four different valleys of Pădurea Craiului Mountains (Table 1, Fig. 1). Five populations of *P. leptodirum* (*P. leptodirum* s. str., *P. l. jeannely*, *P. l. hazayi*, *P. l. moldovani*, *P. l. leptodirum* and *P. l. problematicus*) were collected in two different valleys in Bihorului Mountains (Table 1, Fig. 1).

In each cave, between 10 and 12 individuals were collected for the analysis. The total number of specimens was 145 (89 individuals for the first species and 56 for the second one). Specimens were preserved in 95% ethanol until DNA extraction was processed.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the entire specimens using the DNeasy Blood and Tissue Kit (Qiagen), following the producer's protocol. A fragment of 571 base pairs (bp) of the mitochondrial Cytochrome Oxidase I (COI) gene was amplified using LCO1490 and HCO2198 primers (Folmer et al. 1994).

Double stranded amplifications were performed in a 50 µl reaction volume containing buffer, 5 µl dNTP's 10 mM, 0.5 µl primer 10 mM (each primer), 0.4 µl TAQ polymerase (5U/µl) and 38.6 µl purified water. Each PCR cycle (of a total of 30 cycles) consisted of a denaturation step at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 7 min. PCR products were purified following the manufacturer's protocol for the PCR-Nucleospin Gel and PCR Clean-Up (Macherey-Nagel). Both strands were sequenced on an automated sequencer.

Genetic analyses

Sequences were aligned and edited with BioEdit (v. 7.2), the number of transitions and transversions were analysed with DNAsp (v 5.10.1) (Librado and Rozas 2009). We used MEGA7 (v.7.0) (Kumar et al. 2016) to analyse interspecific haplotype diversity. Genetic structure between populations of the two species or within populations of the same species was analysed with F-statistics using Arlequin (Excoffier et al. 2010) by calculating the following parameters: haplotype diversity (h), the absolute haplotype frequencies, and the nucleotide diversity (π_n). The minimum spanning network was built using PopArt software (Leigh and Bryant 2015).

Mean pairwise intra- and interspecific distances were determined using MEGA (Tamura et al. 2013). Analysis was conducted using uncorrelated p-distance. The analysis involved 145 nucleotide sequences. All codon positions were included.

Using PAST software (Hammer et al. 2001) a Mantel test (Mantel 1967) with 5,000 simulations was carried out to test for an isolation-by-distance (IBD) signature (a positive correlation between geographic and genetic distances (Wright 1943; Slatkin 1993).

The hierarchical distribution of genetic variation was characterized using analysis of molecular variance (AMOVA). This method apportions genetic variation within and among groups, estimating Φ-statistics (Weir and Cockerham 1984; Excoffier et al. 1992; Weir 1996) that are analogous to Wright's hierarchical fixation indices (FST) under the island model of gene flow (Wright 1951). Three-level AMOVA was conducted in ARLEQUIN 3.5.1.2 (Excoffier et al. 1992, 2005) using an FST-like estimator (Fixation Index). For each of the two considered species, AMOVA was run three times considering different groups of populations on the basis of the geological and geographic characteristics. In particular, samples were partitioned by the river basin (Table 1), populations within geographic regions and inside each population. The tests included permutation of inferred haplotypes among groups (FCT); individual haplotypes among populations but within group (FSC); inferred haplotypes among populations (FST).

In order to test for the monophyly of the two Leptodirini species we carried out phylogenetic analysis within a Bayesian framework. J model test (Dariba et al. 2012) was used to perform a hierarchical likelihood ratio test and calculate approximate Akaike Information Criterion (AIC) values of the nucleotide substitution models.

Phylogenetic analysis was performed using Bayesian inferences as implemented by the software MrBayes 3.2.7 (Ronquist et al. 2012). Two simultaneous searches, comprising four Markov chains (MCMC) each and starting from a randomly chosen tree were run for 1,000,000 generations and sampled every 100 generations.

Convergence on a common phylogenetic topology by separate Bayesian searches was checked using Tracer 1.7 (Rambaut et al. 2018). The effective sample size (ESS) of all parameters showed values above 1,000 (values much higher than the threshold of statistical significance, ESS > 200) in both simultaneous searches, indicating that MCMC had converged. Out of 20,000 trees, the first 1,000 were discarded as burn-in, and posterior probabilities (PP) were calculated from post-burn-in trees. The tree was rooted with a species of Leptodirini from Sardinia, *Ovobathysciola* sp.; we were provided with one specimen.

Results

Sequences were obtained from a total of 145 individuals, 89 for *P. gracile* and 56 for *P. leptodirum* (Table 1).

The alignment consisted of 571 bp and defined 46 variable sites, of which 44 were parsimony informative. The nucleotide diversity among all sequences was π_n = 0.033. The sequences are deposited in Genbank with the Accession Numbers OL457148–OL457159 (for H1–H11 and *Ovobathysciola*, respectively).

Intraspecific variability

We identified seven haplotypes for *P. gracile* (numbered H1–H7; Table 1), separated from each other by a maximum of eight mutations, whose geographic distribution is represented in Fig. 2. The haplotypes were separated in two haplogroups according to the genetic divergence between them. One group consisted of specimens of Doboş (DOB), Valău (VAL), Ciur-Ponor (CPO) from Albioara Valley/Sohodol basin, and Vizu II (VIZ) from Vida Valley/Holod basin. The second group was represented by Cubleş (CUB) from Vida Valley/Holod basin, Tocoș (TOC)/Sohodol basin, Întorsuri (INT) from Runcșor Valley/Runcșor basin, and Gruieţ (GRU) from Șteazelor Valley/Roșia basin.

The most widespread haplotypes were H1 and H3, each showing a frequency of 40% in all the analyzed specimens. In particular, individuals from Ciur-Ponor and Doboş caves were fixed for H3 haplotype that was also identified in two other caves, Valău (75%) and Vizu II (42%). Haplotype H1 was shared by the individuals from Cubleş (82%), Gruieţ (82%), Întorsuri (82%), and Tocoş (73%) caves. Haplotypes H5 and H7 were identified in a single specimen, each from Vizu II and Tocoș caves, respectively.

Haplotypes H6 and H4, present with a frequency of 50% and 25%, appeared to be exclusive of Vizu II and Vălău caves, respectively. Haplotype H2 was spread in four caves, Cubleş, Gruieţ, Întorsuri, and Tocoş, with a frequency of 18% in each case. The haplotype diversity for *P. gracile* was *Hd* = 0.684 and nucleotide diversity was π_n = 0.003.

The haplotype network for *P. gracile* is illustrated in Fig. 3a. The identified haplotypes are divided in two clusters: one comprising haplotypes H1, H2 and H7, and

Table 2. *P.* (*Parapholeuon*) *gracile* – computing conventional FST from haplotype frequencies (values in bold indicate significance at the 0.05 level after Bonferroni correction).

	CUB	VIZ	TOC	INT	CPO	DOB	VAL
VIZ	**0.52155**						
TOC	-0.07556	**0.45111**					
INT	-0.10000	**0.52155**	-0.07556				
CPO	**0.82896**	**0.43893**	**0.75370**	**0.82896**			
DOB	**0.83636**	**0.45372**	**0.76364**	-0.10000	0.00000		
VAL	**0.63047**	**0.25069**	**0.56005**	**0.63047**	0.15691	**0.63047**	
GRU	-0.10000	**0.52155**	-0.07556	-0.07556	**0.82896**	**0.83636**	**0.63047**

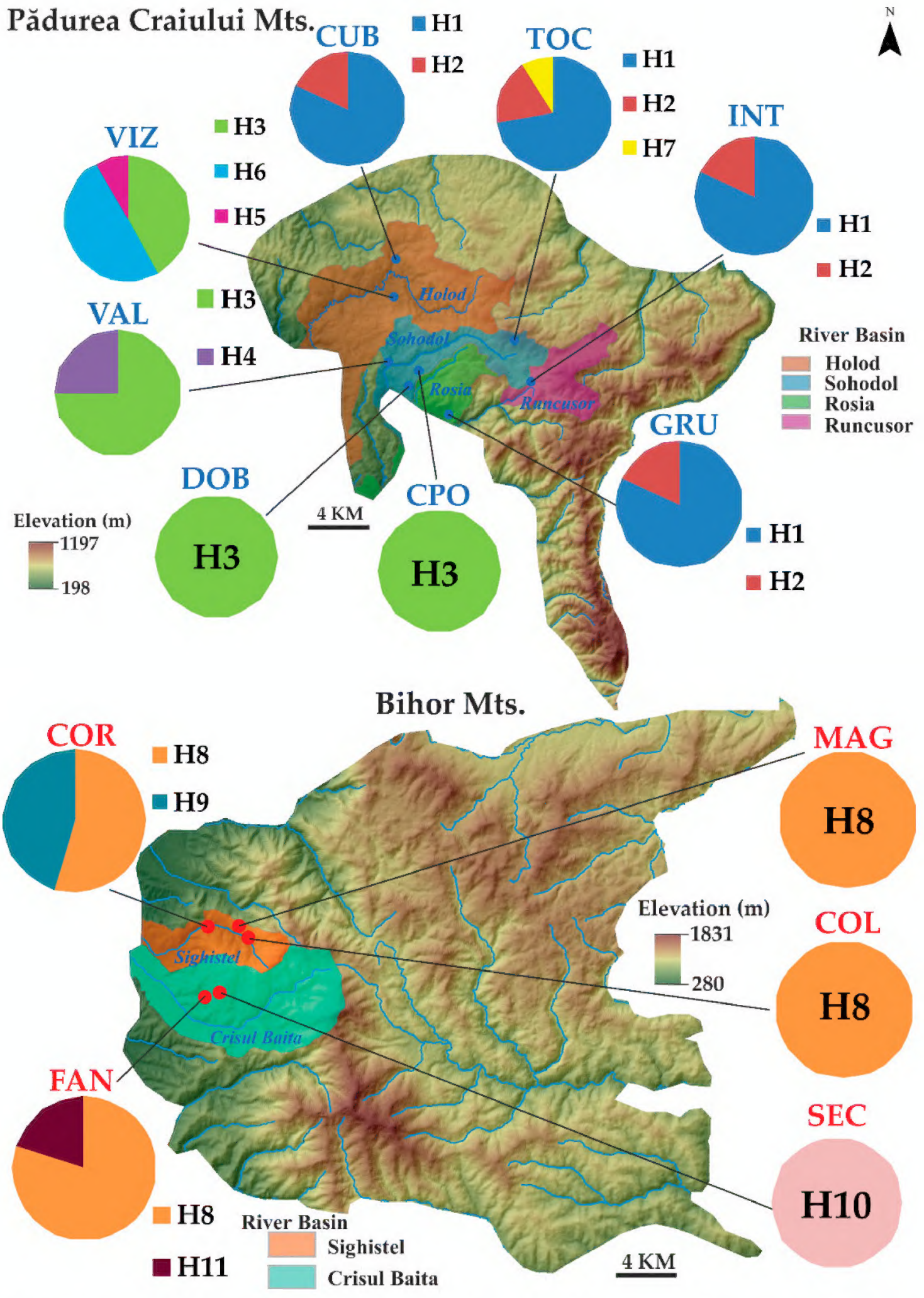

Figure 2. Geographic distribution of haplotypes in *P. gracile and P. leptodirum*. Individuals of DOB and CPO caves are fixed for the haplotype H3 in *P. gracile*; individuals of SEC cave are fixed for the unique haplotype H10 in *P. leptodirum* (abbreviations for the names of caves as in Table 1).

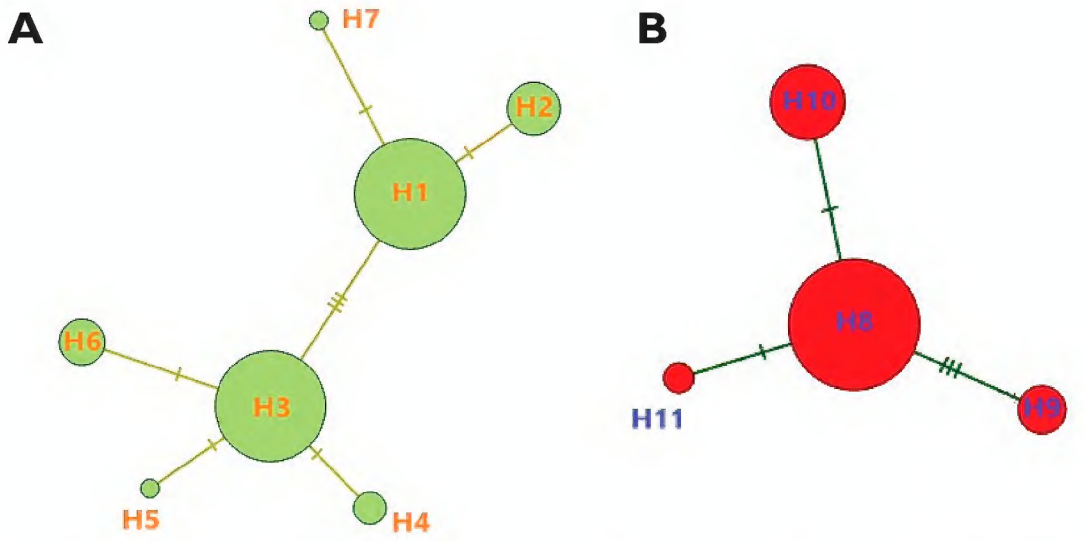

Figure 3. Minimum spanning networks **a** for *P. gracile* with haplotypes H1–H7 and **b** for *P. leptodirum* with haplotypes H8–H11. Multiple mutational steps between haplotypes H1–H3 and H8–H9 could be either un-sampled haplotypes or extinct ones. Haplotype numbers are as in Table 1.

the other haplotypes H3–H6. The two clusters are differentiated by three mutations. Within each cluster, the haplotypes are separated by only one mutation.

FST values for *P. gracile* are presented in Table 2, in which 19 of 28 inter-populational comparisons provided significance at 0.05 level of probability. All negative values indicated a lack of genetic differentiation between the respective populations. FST values over 0.80 indicated a certain degree of genetic differentiation and reduced gene flow between populations included in the two clusters. Mean p-genetic uncorrelated distances between populations belonging to *P. gracile* ranged between 0.1 and 0.9%.

Analysis of molecular variance (AMOVA), suggested some degree of genetic structure within each population (FST = 0.856, P = 0). Genetic variation among different geographic groups and among populations within each clade was 49.4% and 36.2%, with FCT = 0.493 (P > 0.05) and FSC = 0.715 (P = 0), respectively. Mantel test did not suggest a clear isolation by distance across the sampled region (R2 = -0.002, P > 0.05).

In *P. leptodirum* only four haplotypes have been identified (numbered H8–H11; Table 1), separated from each other by a maximum of five mutations, whose geographic distribution is shown in Fig. 2. The most frequent haplotype was H8, spread in all the analyzed cave populations, except for Secătura Cave. In this case, all sampled individuals were fixed for haplotype H10. In particular, samples from Coliboaia and Măgura caves were fixed for haplotype H8 that was also identified in Fânaţe (80%) and in Corbasca (55%) caves. Moreover, Fânaţe and Corbasca caves showed also haplotype H11 and H9 with a frequency of 20% and 45%, respectively. The haplotype diversity for *P. leptodirum* is *Hd* = 0.517 and nucleotide diversity was π_n = 0.001. The haplotype network for this species is illustrated in Fig. 3b.

For the genetic structure of *P. leptodirum*, the indicators of genetic population structure (FST) are presented in Table 3; of 10 inter-populational comparisons, 7 provided significance at the 0.05 level of probability. The highest FST values (>0.70) were for comparisons between the population from Secătura Cave with the other four

Table 3. *P.* (s. str.) *leptodirum* – computing conventional FST from haplotype frequencies (values in bold indicate significance at the 0.05 level after Bonferroni correction).

	COL	MAG	COR	FAN	SEC
MAG	0.00000				
COR	**0.40000**	**0.41463**			
FAN	0.12438	0.13669	**0.19767**		
SEC	**1.00000**	**1.00000**	**0.73742**	**0.83762**	

populations, indicating a degree of genetic differentiation between populations and a limited level of gene flow.

Mean genetic distance between populations belonging to *P. leptodirum* ranged from 0.1 to 0.7%.

Analysis of molecular variance (AMOVA) suggested, also in this case, some degree of genetic structure within the populations (FST = 0.623, P = 0). Genetic variation among different geographic groups and among populations within each group was 11.9% and 50.4%, with FCT = 0.119 (P > 0.05) and FSC = 0.572 (P = 0), respectively. Mantel test did not suggest a clear isolation by distance across the sampled region (R2 = -0.002, P > 0.05).

Interspecific variability

As expected, genetic variation between *P. gracile* and *P. leptodirum* was much greater than intraspecific variation, with a mean genetic distance of 6.55%. Analysis of molecular variance (AMOVA) carried out considering the taxonomic assignment of each population and not the valley, suggested that the two species are well differentiated showing a genetic variation of 95.6%, with FCT = 0.955 (P = 0.002).

The phylogenetic analysis, carried out considering TrN + G model, as suggested by J model test, strongly supports the monophyly of the two species and their clear genetic separation. *P. leptodirum* showed a certain degree of homogenization among its populations, although each was described as a different subspecies. On the other hand, *P. gracile* showed a higher genetic divergence between the analyzed populations, forming two distinct clades. However, in this case the subspecies were not genetically supported, since Cubleș (*P. g. gracile*) and Vizu (*P. g. bokorianum*) did not form different clades, but are linked to the other populations, representing *P. g. chappuisi* (Fig. 4).

Discussion

The genetic variability at COI DNA barcode detected in populations of both analyzed species of *Pholeuon* is quite low. We found a maximum of eight mutations between haplotypes of *P. gracile* and a maximum of five mutations separating the haplotypes of *P. leptodirum*. This result agrees with other studies concerning the analysis of COI DNA barcode in cave dwelling species. Intraspecific genetic variation in seven

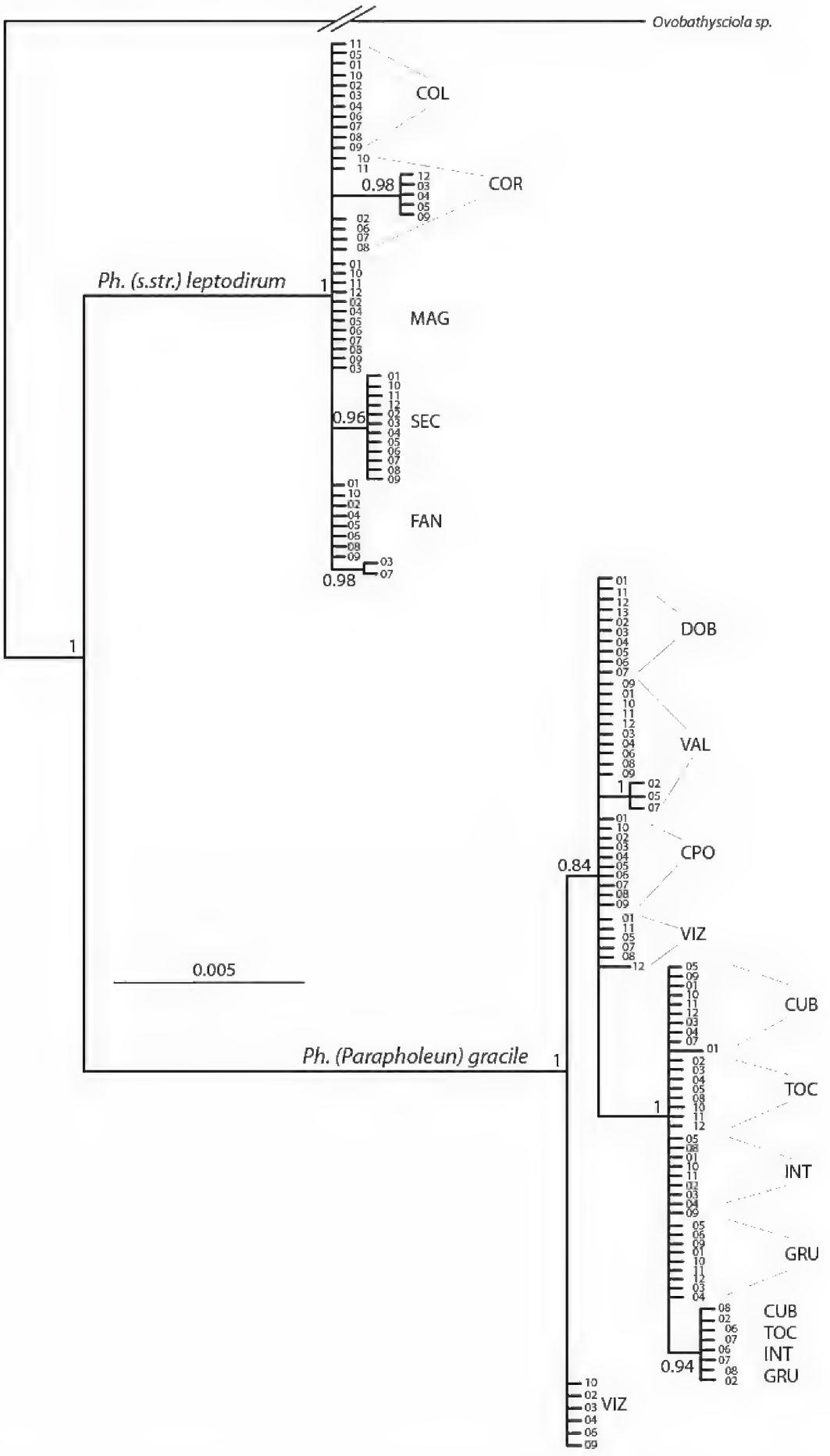

Figure 4. Bayesian tree constructed from 145 individuals of *P. gracile* and *P. leptodirum* from the Apuseni Mountains, belonging to 13 populations (caves). The genetic separation of the two species is clear. As outgroup an *Ovobathysciola* sp. from Sardinia was used.

species of *Bathysciola* (Coleoptera, Leiodidae, Leptodirini) from Central-Southern Italian Apennines and Pre-Apennines ranged from 0 to 1.5% (Latella et al. 2017), while in *Dolichopoda* cave crickets (Orthoptera, Rhaphidophoridae) intraspecific genetic variation ranged from 0 to 1% (Allegrucci et al. 2005, 2014, 2021). The investigation

of two species of the *Tetracion* troglobitic millipede (Diplopoda, Callipodida, Abacionidae) revealed a maximum of 1.4% intraspecific genetic divergence (Loria et al. 2011). Also, populations' COI genetic divergence levels in the troglobitic *Darlingtonea kentuckensis* (Coleoptera, Carabidae, Trechinae) were found at 1.3% (Boyd et al. 2020).

However, despite the low variability, the studied cave populations showed a significant level of genetic structure. AMOVA analysis evidenced significant partitioning of variation within and among populations in both studied species. This result is not surprising for troglobionts because it reflects the possible barriers between the different caves and/or groups of caves to which populations are confined. On the other hand, genetic variation is not significantly partitioned among geographic groups in both species (FCT shows $P > 0.05$ in both cases) and Mantel test did not show a phylogeographic pattern. These results could be explained by the evolution of caves both in Pădurea Craiului Mountains inhabited by *P. gracile* and in Bihorului Mountains where *P. leptodirum* is found.

The formation of the caves is strongly related to hydrological network development and the tectonics, both at regional and local scales. The area in Pădurea Craiului Mountains where the caves are located is a highly tectonic region (Orașeanu 2020), with several stages of cave systems evolution. Caves can evolve quite differently from one another regarding water input, rock type, geotectonic features, and local hydrological system although being part of the same basin (Rusu 1981).

The hydrographic network of Pădurea Craiului Mountains is not well organized due to very intense processes of karstic caption (Orășeanu 2020) that promotes the formation of geological and hydrographic barriers, at the same time preventing gene flow between cave populations. For example, Vizu and Cubleş caves, on one hand, and Doboş/Ciur Ponor and Valău caves, on the other hand, are located in the same valley, but on the opposite slopes. Vizu Cave population showed a haplotypic composition (H3, H5, H6) completely different from Cubleș Cave (H1 and H2). The two caves are located on different slopes of the same valley, less than 1 km apart from each other. Cubleş and Vizu caves could belong to different stages of evolution and development of the Vida River (Orășeanu 2020). Both of them were carved by tributary streams (Cubleş by Blajul and Vizu by Viduța), controlled by the local water table (e.g., different incision rates). Because the relative altitude varies for the two caves, with Cubleş at ~50 m and Vizu at ~2 m above the present waterflow the river Viduţa might act as a hydrographical barrier even during the incision of the valley and, therefore, could promote genetic isolation and differentiation of populations. We cannot assume that the paleogeographic changes were the only factors, but we can hypothesise that these could be the first step towards the isolation of populations. Ciur Ponor and Doboș caves are located on the same side of Albioara valley, specimens of the two populations share the same haplotype, H3. In Vălău cave population, located on the opposite slope, haplotype H3, but also the unique haplotype H4 have been identified. Since, in both cases, caves differ in aplotype composition, the rivers could act as hydrographic barriers, preventing the dispersal of populations. On the contrary, Tocoș, Întorsuri and Gruieț populations share haplotypes, as no hydrographic barrier separates these caves (Table 1, Fig. 2).

Bihorului Mountains, with caves hosting *P. leptodirum*, are mostly comprised by limestones, dolomites, conglomerates, and eruptive rock (Seghedi 2004). Still, the

development of the karst network in Bihorului Mountains is characterized by intense fragmentation of the carbonate rocks with the development of large-scale karst systems and a petrographic mosaic shaping the relief. Populations of Măgura/Coliboaia caves, located on the same side of the valley, share the same haplotype (H8). At the same time, specimens from the Corbasca cave, located on the opposite slope, have the haplotype H8 and the unique haplotype H9. The populations from Fânațe and Secătura caves are situated on the same side of Bulzului valley. The caves formed in the same type of limestone are geographically close (~1 km). The first cave is inhabited by *P. l. leptodirum*, while the second one by *P. l. problematicus*. Their haplotypic composition is completely different, with Fânațe Cave population showing the most common haplotype H8 and the unique haplotype H11 in low frequency, while Secătura Cave population is fixed for the unique haplotype H10, suggesting a complete lack of gene flow. Although the two caves have certain similarities and are located at different altitudes (Secătura at 740 m.a.s.l. and Fânațe at 580 m.a.s.l.), they could have been populated at different stages by the hypothetical ancestral populations that were already genetically differentiated.

All these local features could establish geologic and hydrogeologic barriers, even for caves that are geographically close. So, even the smallest change at a certain time, in the local evolution of a cave could, be a limiting factor for the dispersal of cave populations (Sánchez-Fernández et al. 2018). Different slopes of the same river valley could represent a geographic barrier strong enough to hamper gene flow in species with poor dispersal capabilities. This could potentially be even reinforced when unsuitable habitats (i.e. impermeable strata, dry conditions, small voids etc) must be crossed. This assumption needs to be validated with additional analyses conducted on a more extensive sampling design, that would include multiple basins with multiple caves on the opposing valley slopes.

In conclusion, in both of the analyzed species, *P. gracile* and *P. leptodirum*, the genetic divergence of the COI DNA is too low to discriminate between different subspecies, although in some cases a certain degree of intraspecific genetic structure has been found (for example, between the proposed subspecies *P. g. bokorianum* and *P. l. problematicus*), suggesting that a reassessment of their status is needed. This result was expected because the genetic divergence at DNA barcoding is not informative about the species status when recently diverged species are compared and complete lineage sorting has not yet been achieved (DeQueiroz 2005; Lencioni et al. 2021). Episodes of gene flow between the different proposed subspecies represent a possible explanation preventing complete lineage sorting and most of the populations belonging to the two considered species here are not completely isolated. Moreover, the identification of species based on one single genetic marker can be incongruent with species identification using morphological characters (Moritz and Cicero 2004; Matz and Nielson 2005; Allegrucci et al. 2014; Lencioni et al. 2021).

In the Bayesian analysis of the relationships between the analyzed taxa, the two *Pholeuon* species are monophyletic and well differentiated from each other, with *P. gracile* showing a higher differentiation than *P. leptodirum*. As far as interspecific variation is concerned, the mean genetic distance was between 6.4 and 7.7%, with a mean value of 6.55%. Following Hebert et al. (2003) COI divergence ranges from

below 1% to 16–32% between species of beetles within the same genus with an average sequence divergence of 11.2. Our value falls within the intermediate range, suggesting that the two species are well differentiated and possibly for a long time. It is not rare to find high genetic differentiation between species of troglobionts as a result of allopatric speciation and the formation of hydro-geographic barriers due to changes in the surface landscape along the different climatic periods. Leptodirini appears to be an ancient cave group, as demonstrated from previous papers (Caccone and Sbordoni 2001; Ribera et al. 2010; Latella et al. 2017) with different species separated for long periods of time and accumulating genetic divergence. High genetic differentiation has been found also in a group of nine species of *Bathysciola*, where the interspecific genetic distances ranged from 3.1% of up to 15.1% (Latella et al. 2017). In this case, the two main lineages of *Bathysciola* were considered and divergence times suggested a Miocene deep cladogenesis. The two clades were shaped by the geological events during the Pliocene and the climatic changes of the Pleistocene (Latella et al. 2017). On the other hand, low levels of mitochondrial diversity have been found in species of the *Pholeuon* genus belonging to *Drimeotus* phyletic lineage although they showed deep cladogenesis with species belonging to the *Drimeotus* genus, included in the same phyletic lineage and revealing a possible split in the late Miocene (Bucur et al. 2003).

In conclusion, based on the observed genetic structure between the different populations of the two *Pholeuon* species further studies including more populations and species are needed to understand the genetic variation patterns of the group and provide valuable information for the life histories and conservation of Leptodirini in Romania. The data presented in this contribution – albeit preliminary in terms of sampling and limited in terms of genetic markers– confirm the importance of subterranean environment as a reservoir of biodiversity at a microgeographical scale. Such biodiversity should be hence managed accordingly.

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